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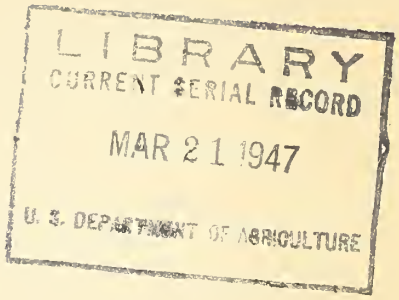

Quantitative Determination of Extractable Gossypol in Cottonseed and Cottonseed Meal *

A Spectrophotometric Method

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The reaction of gossypol with antimony trichloride in ether and chloroform extracts of cottonseed is specific and the reaction product is sufficiently stable to permit accurate spectrophotometric determination of the gossypol concentration of such extracts. A rapid direct method for determination of free gossypol in cottonseed is reported, in which the gossypol is extracted by equilibrating ground cottonseed and chloroform, treating the extract with concentrated hydrochloric acid, and applying the antimony trichloride reaction directly to the treated extract.

IT WAS recently shown (*1*) that gossypol reacts with antimony trichloride in chloroform to form a soluble red product having an absorption curve which exhibits a broad maximum at 510 to 520 mμ. It was shown further that the magnitude of the extinction at the maximum is directly proportional to the concentration of gossypol in the test solution and that therefore the absorption spectrum of the antimony trichloride reaction product could be used as a quantitative measure of gossypol in solution. However, it was found that the absorption curves of the antimony trichloride reaction products with cottonseed extracts differed somewhat from those of similar products obtained with solutions of pure gossypol.

Subsequent investigation of the antimony trichloride reaction products with cottonseed extracts has shown that in most cases the reaction product exhibits, initially, an absorption curve identical with that obtained with pure gossypol. Antimony trichloride evidently reacts more rapidly with gossypol than with the interfering reactants which may be present in cottonseed extracts. Consequently, when the absorption maximum at 510 to 520 mμ of the reaction product of antimony trichloride with a cottonseed extract is determined before interfering reactions have developed, the height of this maximum serves as a quantitative measure of the concentration of gossypol in such an extract.

In some hydraulic press-cake meals, as well as in some raw cottonseed, much of the "free" gossypol occurs in the form of an orange-colored pigment (*2*) which can be converted into gossypol by treating the extracts with concentrated hydrochloric acid. Following such treatment, these extracts react with antimony trichloride in a manner completely analogous to that of cottonseed extracts containing negligible amounts of the orange-colored pigment.

ANTIMONY TRICHLORIDE REACTION WITH PURE GOSSYPOL

The gossypol–antimony trichloride reaction is carried out as follows:

To 1 ml. of a chloroform solution of purified gossypol in a glass-stoppered absorption cell there are added 1 drop of acetic anhy-

[1] On military leave for duty with U.S.N.W.R.

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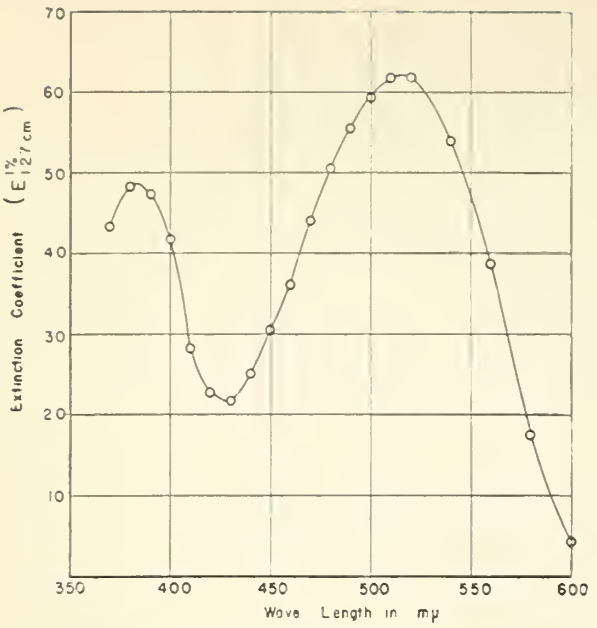

Figure 1. Absorption Curve of Antimony Trichloride Reaction Product with Pure Gossypol

dride and 5 ml. of a saturated chloroform solution of antimony trichloride. The acetic anhydride is added to prevent the development of the hazes produced by moisture in the extracts. For the same reason it is desirable to mix the reagents directly in the absorption cells and thus avoid unnecessary exposure to atmospheric moisture. The transmission of the solution is measured against that of a blank which consists of 1 ml. of chloroform, 1 drop of acetic anhydride, and 5 ml. of a saturated chloroform solution of antimony trichloride.

The volumes of reagents indicated were chosen to suit the capacity of the cells designed for the Coleman double monochromator spectrophotometer which was used for all absorption measurements reported here. The Coleman spectrophotometer is equipped with cells having an estimated optical depth of 1.27 cm. and all extinction coefficients were, therefore, expressed in terms of this optical depth rather than the customary 1.0-cm. depth. Extinction coefficients are defined by the equation $E = \frac{\log I_0/I}{cl}$ where $\log I_0/I$ is the extinction, I_0, the intensity of light transmitted by the blank, I the intensity of light transmitted by the solution, c the concentration of the solution, and l the length of the path of light through the liquid. The extinction coefficients were calculated in terms of the optical depth of the absorption cells used (1.27 cm.) and, for convenience in calculating the concentration of gossypol in test extracts, they were expressed in terms of the concentration in grams of gossypol per 100 ml. of the original solution to which the acetic anhydride and the antimony trichloride solution were added.

The absorption curve of the antimony trichloride reaction product with pure gossypol is shown in Figure 1. The curve is characterized by two maxima, a broad one in the visible range at 510 to 520 mμ, and a sharper one in the near ultraviolet at 380 mμ, and by a minimum at 430 mμ.

As shown in Figure 2, a straight-line relationship exists between the values of $\log_{10} I_0/I$ and the concentration of gossypol at 520, 430, and 380 mμ for concentration of gossypol in the original solution ranging from 0.004 to 0.016 gram per 100 ml. of solution. These values were obtained with solutions of three preparations of gossypol prepared according to two independent procedures (*1*, *2*).

The existence of two well-defined, widely separated absorption maxima permitted the mathematical characterization of the absorption spectrum (*4*, *15*) of the gossypol–antimony trichloride reaction product in terms of the ratios of the magnitudes of the maxima to each other and to the minimum. These ratios can be used for establishing the specificity (*4*, *15*) of the reaction for gossypol contained in extracts of cottonseed. As shown in Table I, the value of the ratio R_a, $\log_{10} I_0/I$ at 520 mμ to $\log_{10} I_0/I$ at 430 mμ, is 2.68 ± 0.23, and the ratio R_b, $\log_{10} I_0/I$ at 520 mμ to $\log_{10} I_0/I$ at 380 mμ, is 1.22 ± 0.07 for pure gossypol in the antimony trichloride reaction.

The absorption spectrum in the range 370 to 600 mμ reaches its maximum development within 10 minutes after the reagents are mixed and is stable for at least 24 hours.

In contrast to the reaction of gossypol, the orange-colored pigment from cottonseed (*2*) does not form a stable reaction product with antimony trichloride. When a chloroform solution of the orange-colored pigment is first treated with concentrated hydrochloric acid and then reacted with antimony trichloride, the reaction product exhibits an absorption spectrum identical with that of the gossypol–antimony trichloride reaction product with respect to both positions and ratios of maxima and minimum.

Because of its broadness and its position in the visible wavelength range, the absorption maximum at 510 to 520 mμ serves best as a standard for computing gossypol concentrations in test solutions. When dilutions ranging from 0.004 to 0.016% gossypol of nine different solutions of three preparations of pure gossypol obtained by two different procedures (*1*, *2*) were used, the values for $E^{1\%}_{1.27\,cm.}$ at 520 mμ shown in Table I were obtained. From these data it may be concluded that the value of $E^{1\%}_{1.27\,cm.}$ at 520 mμ is 65.5 ± 1.9 for gossypol in the antimony trichloride test where the concentration of gossypol is expressed in terms of grams per 100 ml. of the original solution.

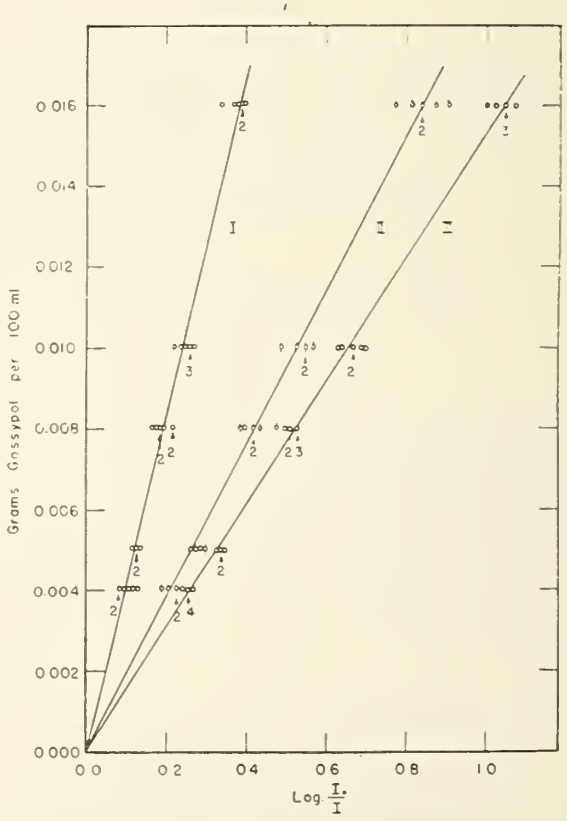

Figure 2. Demonstration of Beer's Law in Gossypol–Antimony Trichloride Reaction

I. Extinction at 430 mμ *vs.* gossypol concentration
II. Extinction at 380 mμ *vs.* gossypol concentration
III. Extinction at 520 mμ *vs.* gossypol concentration
Arabic numerals indicate multiplicity of points obtained in independent tests.

Table I. Absorption Spectrum of Antimony Trichloride Reaction Product with Pure Gossypol

Gossypol Preparation	Concentration, Gram/100 Ml.	R_a[a]	R_b[b]	$E\,^{1\%}_{1.27\text{ cm.}}$ at 520 mμ
FSBP	0.010	2.49	1.17	63.8
	0.010	2.88	1.29	62.9
	0.016	2.72	1.15	62.5
	0.008	3.16	1.29	63.7
	0.008	2.84	1.28	61.9
	0.004	3.02	1.37	65.0
Y_{00}	0.010	2.69	1.22	69.9
	0.005	2.49	1.20	68.4
	0.010	2.69	1.26	65.8
	0.005	2.57	1.20	67.4
	0.005	2.79	1.24	64.6
	0.016	3.03	1.32	63.9
	0.008	2.82	1.24	64.5
	0.004	2.49	1.15	65.0
FSB[c]	0.016	2.80	1.27	66.9
	0.016	2.77	1.16	65.4
	0.016	2.66	1.29	65.4
	0.008	2.58	1.21	63.6
	0.008	2.53	1.15	66.3
	0.008	2.47	1.28	66.3
	0.004	2.11	1.11	63.0
	0.004	1.99	1.08	65.0
	0.004	2.68	1.34	65.0
	0.010	2.69	1.26	68.8
	0.010	2.61	1.19	66.8
	0.010	2.86	1.19	66.8
	0.005	2.56	1.10	65.6
	0.005	2.69	1.22	67.4
Y_{00}[c]	0.016	2.73	1.24	65.4
	0.008	2.83	1.22	66.3
	0.004	2.96	1.27	66.0
Average values		2.68	1.22	65.5
Deviation from mean		±0.23	±0.07	± 1.9

[a] $R_a = \log_{10} I_0/I$ at 520 mμ to $\log_{10} I_0/I$ at 430 mμ.
[b] $R_b = \log_{10} I_0/I$ at 520 mμ to $\log_{10} I_0/I$ at 380 mμ.
[c] Solutions treated with concentrated hydrochloric acid before reaction with antimony trichloride.

ANTIMONY TRICHLORIDE REACTION APPLIED TO COTTONSEED EXTRACTS

The complete absorption curves, in the region of 370 to 600 mμ, of freshly prepared mixtures of chloroform extracts, and of chloroform solutions of ether extracts, of many cottonseeds with antimony trichloride are almost identical with the absorption curve obtained with pure gossypol and antimony trichloride. As the reaction mixtures stand, absorption increases in the shorter wave-length region and the whole character of the curves changes. In order to establish the specificity of the reaction it was necessary to read the spectra as soon as possible after the reagents were mixed. Therefore, only the absorptions at the critical wave lengths, 530, 520, 510, 500, 430, 390, 380, and 370 mμ, were determined and these were read as rapidly as possible after the expiration of the 10-minute induction period. With this procedure it was found, as shown in Table II, that the absorption spectra of fresh mixtures of antimony trichloride with chloroform solutions of both chloroform and ether extracts of cottonseed are analogous to the gossypol–antimony trichloride absorption spectrum. The maxima and the minimum occur at the same wave lengths and are of the same relative magnitudes, as shown in Table II. The spectra listed in the table were chosen to illustrate both the best and worst agreement of the R_a and R_b values of the extract reaction products with those of the pure gossypol reaction product. Several examples of values obtained from the reaction performed with duplicate samples of the same extract and with duplicate extracts of the same cottonseed are included. The duplication of the values of E at 520 mμ, even though the values of the ratios are not duplicates, demonstrates that the value of E at 520 mμ is an accurate measure of the gossypol concentration.

Chloroform and ether extracts of some hydraulic-pressed cottonseed meals, as well as of some raw cottonseed, produce an orange or yellow color instead of the characteristic red reaction product with antimony trichloride. The absorption spectra of these reaction products are very unstable but show certain definite tendencies as illustrated in curves 1 and 2 of Figure 3. The first maximum shifts from 510 to 520 mμ to 490 mμ; a new maximum appears at 450 mμ; and the maximum at 380 mμ shifts to 390 mμ. Such curves appear to represent the result of superposing on the gossypol–antimony trichloride absorption curve, which has maxima at 380 mμ and 510 to 520 mμ, the absorption curve of the unstable reaction product of antimony trichloride with the orange-colored pigment (*2*), which has a maximum at 450 to 460 mμ, and that of the unstable antimony trichloride reaction product with at least one other pigment, which has maxima at 390 and 490 mμ. In confirmation of this explanation of the shape of these curves, it was observed that when such extracts were treated with concentrated hydrochloric acid prior to their reaction with antimony trichloride, reaction products which showed typical gossypol–antimony trichloride absorption curves were obtained, as shown in Table II.

Thus it has been possible to apply absolute criteria for the establishment of the specificity (*4, 15*) of the antimony trichloride reaction for gossypol in cottonseed extracts. It is evident that any concurrent reactions of antimony trichloride to form colored products with extractable components of cottonseed other than gossypol would alter the shape of the curve and thus change the values of the ratios. In the absence of such interfering reactions the absorption serves as a direct measure of the gossypol concentration in the extracts.

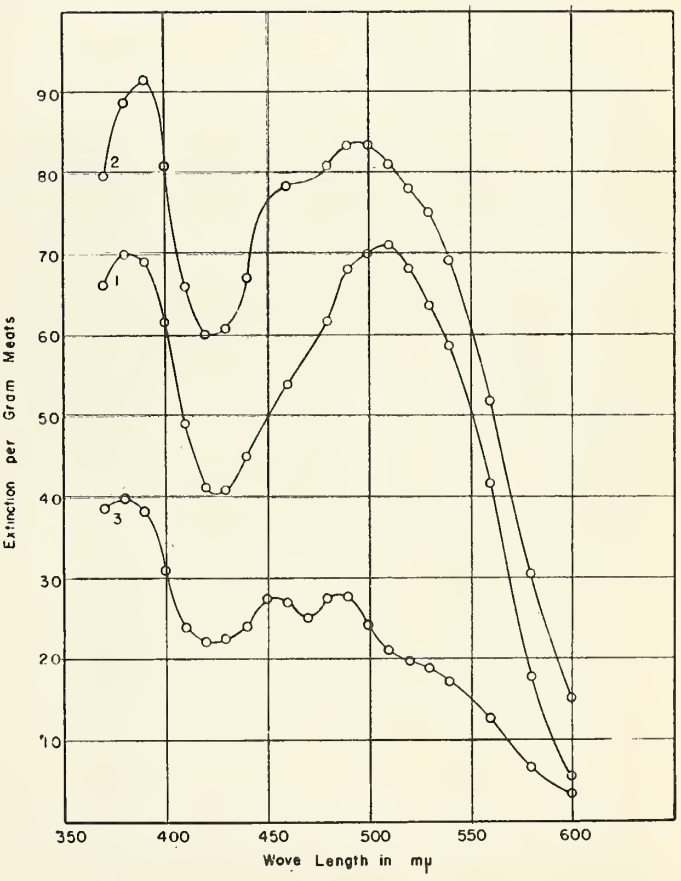

Figure 3. Absorption Curves of Antimony Trichloride Reaction Products with Cottonseed Extracts

1. Reaction product with chloroform extract 15 to 20 minutes after mixing reactants
2. Reaction product with chloroform extract 24 hours after mixing reactants
3. Reaction product with Skellysolve F extract 24 hours after mixing reactants

Table II. Absorption Spectrum of Antimony Trichloride Reaction Product with Gossypol in Cottonseed Extracts

Cottonseed Sample	Extraction Solvent	R_a	R_b	E at 520 mμ per Gram	Per Cent Gossypol
C-77-I	$CHCl_3$	1.93	1.06	56.2[a]	0.858
	$CHCl_3$	2.07	1.07	56.2[a]	0.858
C-77-VII	$CHCl_3$	1.59	0.90	64.8	0.991
	$(C_2H_5)_2O$	2.21	1.13	65.5	1.00
C-77-IX	$CHCl_3$	2.00	1.13	66.3	1.01
	$(C_2H_5)_2O$	2.14	1.18	66.3	1.01
205e[b]	$CHCl_3$	1.64	1.10	22.5	0.344
	$(C_2H_5)_2O$	1.71	0.95	22.5	0.344
PC-C-77-VIIa[c]	$CHCl_3$	1.23	0.92	15.1	0.235
PC-7[c]	$CHCl_3$	1.78	1.04	32.1	0.481
205a[b]	$CHCl_3$	1.98	1.15	84.2	1.29
PC-9[c]	$CHCl_3$	1.94	1.12	70.0	1.07
105b	$CHCl_3$	2.04	1.17	67.2	1.03
C-78	$CHCl_3$	2.29	1.19	45.2	0.681

[a] Duplicate tests of same extract.
[b] Raw cottonseeds contained considerable quantities of orange-colored pigment, so that chloroform solutions were treated with concentrated hydrochloric acid prior to reaction with antimony trichloride.
[c] Hydraulic-pressed meals treated as in [b].

Fortunately, whether because of compensation or a slower development of interference at this point, within 10 minutes of mixing the antimony trichloride solution with the extract the absorption at 520 mμ increases to a value which remains constant for a considerable period of time, ranging from 24 hours for some extracts to a minimum of 30 minutes for the least stable of a series of 117 extracts of 64 different raw cottonseeds and meals examined. Therefore, the value of the extinction at 520 mμ of the antimony trichloride reaction products with chloroform solutions of ether or chloroform extracts of cottonseed, or with acid-treated chloroform solutions of ether or chloroform extracts of hydraulic-pressed cottonseed meals determined within 10 to 40 minutes after the reagents are mixed, is a true measure of the gossypol content of the extract.

As is shown in Table III, the addition of measured amounts of pure gossypol to cottonseed extracts produces increases in the extinction at 520 mμ of the reaction product with antimony trichloride which are quantitatively proportional to the amount of pure gossypol added. The values shown in Table III establish the precision of the method. When conducted as described, the antimony trichloride reaction method permits the determination of the gossypol content of ether and chloroform extracts of cottonseed and of hydraulic-pressed cottonseed meals with a duplicability within ±1% of the total gossypol concentration of the extracts. This is the limit of precision of the spectrophotometer as it is used in the test.

Absorption spectra curves of antimony trichloride reaction products with cottonseed oils or Skellysolve F extracts differ markedly from that of the gossypol–antimony trichloride reaction product. As shown in curve 3 of Figure 3, when the antimony trichloride reaction product of such an extract has stood for some time, well-defined absorption maxima at 380, 450, and 480 to 490 mμ develop. Treatment of chloroform solutions of the oils or Skellysolve F extracts with concentrated hydrochloric acid prior to reaction with antimony trichloride causes the formation of pink reaction products. The absorption spectra curves no longer exhibit a maximum at 450 mμ and absorption at 510 to 520 mμ is increased, but absorption at 390 and 490 mμ remains high, so that the absorption curves only very slightly resemble the gossypol–antimony trichloride absorption curve. These observations indicate the presence of the orange-colored pigment in cottonseed oils and Skellysolve F extracts. They indicate further, however, that the orange-colored pigment occurs in such relatively small amounts that even when it has been converted to gossypol by the action of hydrochloric acid, the gossypol–antimony trichloride reaction is masked by the reaction of the preponderant interfering pigments. Chloroform and ether extracts of nondefatted cottonseed evidently contain some of this interfering substance, but in much lower relative concentrations, so that its only effect is to reduce the stability of the spectrophotometric test.

Since it has been possible to establish the specificity of the antimony trichloride reaction for gossypol in extracts prepared from 64 different samples of raw cottonseed and hydraulic-pressed cottonseed meal, the validity of the method can be considered to be established beyond reasonable doubt. Consequently, it appears superfluous to make a systematic comparison of the present method with other methods for the determination of gossypol.

The spectrophotometric method for the determination of gossypol proposed by Lyman, Holland, and Hale (*8*) suffers from the disadvantage that the dianilinogossypol absorption curve on which the method is based exhibits only one maximum in the visible region. Because of this limitation, the authors are able to state concerning the specificity of their method only that "there appears to be no source of error due to other pigments present in cottonseed meal", and "if there are other substances besides gossypol in cottonseed meal which give color with aniline, these substances must be closely related to gossypol". When various cottonseed extracts are treated with the carbonyl reagent, dinitrophenylhydrazine, the absorption spectra of the reaction mixtures indicate that carbonyl compounds other than

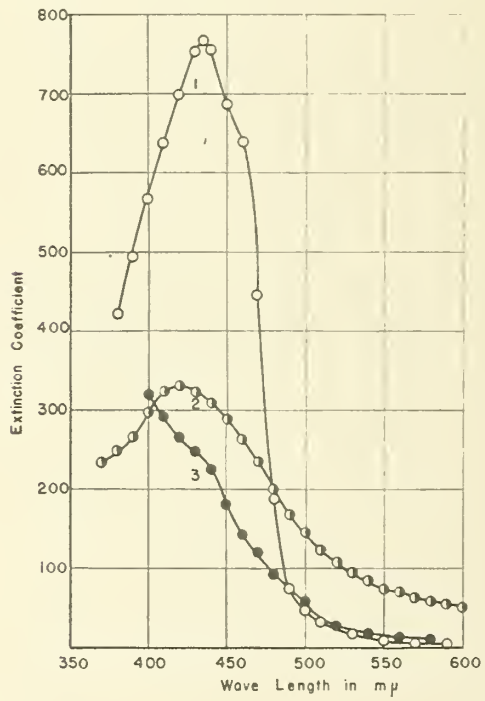

Figure 4. Absorption Curves of 2,4-Dinitrophenylhydrazine Reaction Products with Cottonseed Components

1. 2,4-Dinitrophenylhydrazone of orange-colored pigment of cottonseed
2. 2,4-Dinitrophenylhydrazone of gossypol
3. 2,4-Dinitrophenylhydrazine reaction product with chloroform extract of cottonseed

Table III. Determination of Gossypol Added to Cottonseed Extracts

Sample of Seed or Meal Extracted	Gossypol in Extract	Pure Gossypol Added to Extract	Total Gossypol in Final Mixture	Gossypol Found	Recovery of Gossypol
	G./100 ml.	*G./100 ml.*	*G./100 ml.*	*G./100 ml.*	%
202a	0.00208	0.00416	0.00624	0.00631	101.0
PC-1	0.00516	0.00074	0.00590	0.00599	101.5
	0.00387	0.00147	0.00534	0.00542	101.4
	0.00184	0.00323	0.00507	0.00508	100.2
C-101	0.00184	0.00586	0.00770	0.00760	98.7
	0.00368	0.00439	0.00807	0.00764	94.7
	0.00460	0.00366	0.00826	0.00812	98.3

gossypol and the orange-colored pigment occur in the cottonseed extracts. If these carbonyl compounds also react with aniline, as predicted by Lyman, Holland, and Hale (*8*), they will interfere in the aniline-spectrophotometric method for gossypol whenever they occur in cottonseed extracts. Consequently, no direct comparison of the two spectrophotometric methods was made.

Experience with the aniline precipitation method as modified by Halverson and Smith (*6*) and Smith (*13*) was similar to that recently reported by Lyman, Holland, and Hale (*8*) in that duplicate analyses were obtained with difficulty. No attempt was made to confirm their observation (*8*) that the dianilinogossypol precipitates were frequently impure. On the other hand, the antimony trichloride method nearly always indicated a higher gossypol concentration in a given extract than the aniline precipitation method. This occurred even when the extracts contained no detectable amounts of the orange-colored pigment. In such cases it seems most logical to conclude that the observed discrepancies are due to incomplete precipitation of dianilinogossypol from the extracts. According to a recent report (*10*), the addition of an ether solution of aniline to an ether solution of gossypol results in the precipitation of a mixture of dianilino- and tetraanilinogossypol. If a similar reaction should occur when aniline is added to ether extracts of cottonseed or cottonseed meal, the gossypol content of the extract should not be calculated on the assumption that the precipitate is dianilinogossypol.

Table IV. Time for Complete Extraction of Gossypol from Cottonseed and Cottonseed Meal

Sample	Extraction Solvent	Time, Hours	E at 520 mμ per Gram	Per Cent Gossypol
Cottonseed containing negligible amounts of the orange-colored pigment				
17a	$CHCl_3$	0.5	13.5	0.206
	$CHCl_3$	1	23.1	0.352
	$CHCl_3$	2	34.6	0.528
	$CHCl_3$	2	34.2	0.523
	$CHCl_3$	24	36.1	0.552
C-77-VII	$CHCl_3$	2	64.5	0.975
	$CHCl_3$	24	63.0	0.963
	$(C_2H_5)_2O$	2	65.5	1.00
Cottonseed and hydraulic-pressed meal containing considerable amounts of orange-colored pigment[a]				
PC-77	$(C_2H_5)_2O$	2	11.7	0.179
	$CHCl_3$	2	12.4	0.189
	$CHCl_3$	24	15.1	0.235
	$CHCl_3$	48	15.1	0.235
205e	$(C_2H_5)_2O$	2	16.8	0.257
	$CHCl_3$	2	17.3	0.264
	$CHCl_3$	24	22.5	0.344
	$(C_2H_5)_2O$	24	22.5	0.344
	$CHCl_3$	48	22.8	0.348

[a] All $CHCl_3$ solutions of extracts treated with concentrated HCl prior to reaction with $SbCl_3$.

EXTRACTION OF GOSSYPOL FROM COTTONSEED

With a rapid and accurate method available for the determination of gossypol in cottonseed extracts, the accurate determination of gossypol in cottonseed requires only a reliable and convenient method for extracting gossypol from the seed.

It has been reported that both the duration of extraction and type of extraction apparatus, as well as the moisture content of the seed or hydraulic-pressed meal, affect the amount of gossypol extracted. Most investigators (*5–8*, *11–13*) recommend exhaustive extraction, usually in an apparatus of the reflux type which is designed for thorough rinsing of the substance from which soluble material is extracted. Apparatus of the reflux type must be used when the seed or meal is to be freed entirely from gossypol, or when, as is the case in the use of gravimetric methods, a large amount of gossypol is required for the determination. On the other hand, a simple equilibration can be used effectively when the concentration of extractable gossypol in the seed or meal is to be determined by means of the sensitive spectrophotometric method. The conditions for adequate

Table V. Equivalence of Equilibration and Butt Extraction with Ether

Cottonseed Sample[a]	Kind of Extraction	Time of Extraction, Hours	Ra	E at 520 mμ per Gram Rb	Per Cent Gossypol Seed	Gossypol
C-77-VIII	Equilibration	2	1.97	1.03	61.9	0.945
	Equilibration	24	2.15	1.16	64.6	0.986
	Equilibration	72	1.90	1.04	62.8	0.960
	Butt	24	2.05	1.23	68.8	1.05
	Butt	72	1.95	1.03	68.8	1.05
C-77-IX	Equilibration	24	2.03	1.16	60.3	0.922
	Butt	24	2.03	1.19	62.9	0.960
	Equilibration[b]	2	2.00	1.13	66.3	1.012
	Equilibration	2	2.14	1.18	66.3	1.012

[a] Both series of cottonseed samples contained negligible amounts of orange-colored pigment.
[b] Solvent of equilibration was chloroform.

equilibration can be ascertained by determining what volume of solvent and what contact time must be employed, so that an increase in the proportion of solvent or in the time of contact produces no increase in the concentration of gossypol in solution. An aliquot portion of an extract obtained under these conditions can be used directly in the spectrophotometric method for determining the concentration of extractable gossypol in the seed.

As is shown in Table IV, complete extraction of gossypol from ground cottonseed meats or hydraulic-pressed meal by equilibration with chloroform requires 2 hours when the seed contains negligible amounts of the orange-colored pigment, and 24 hours when the seed or meal contains a considerable concentration of this pigment.

That diethyl ether and chloroform extract equal amounts of the pigments is demonstrated in Tables II, IV, VI, and VIII. Murty, Murty, and Seshadri (*9*) also have reported the equivalence of ether and chloroform for the extraction of gossypol.

In order to confirm the equivalence of simple equilibration and exhaustive extraction, direct comparisons of extracts of replicate samples of the same seeds were made (Table V).

The results of experiments to determine the effect of moisture on the extraction of free gossypol by chloroform and ether are shown in Table VI. These data show, in agreement with the recent report of Murty, Murty, and Seshadri (*9*), that moisture does not play an important role unless it is very low, as in the case of desiccated seed, or so high as to interfere with the "wetting" of the seed by chloroform. Halverson and Smith (*7*) and Lyman, Holland, and Hale (*8*) observed that continued extraction of wet hydraulic-pressed cottonseed meal by wet ether at elevated temperatures gives increased amounts of gossypol. They attributed their results to the absence of a sharp boundary between "free" and "bound" gossypol in cottonseed meal. In view of the results obtained with raw cottonseed, it is more probable that the extraction methods employed by these investigators cause the liberation of some of the bound gossypol. Consequently, for the extraction of free gossypol from cottonseed meal the present authors have employed only the mild conditions of extraction found adequate for the extraction of free gossypol from raw cottonseed.

It is apparent from Table VII that proportions of chloroform to seed varying from 25 to 125 ml. per gram afford complete extraction of gossypol. Consequently, the spectrophotometric method can be used for accurate determination of the gossypol content of seeds of widely varying gossypol content.

In contrast with the consistent results obtained by the equilibration of ground cottonseed kernels and of hydraulic-pressed meal with chloroform and ether shown in the preceding tables and in Table VIII, very erratic results were obtained when the seed was first defatted by extraction with Skellysolve F. As is shown in Table VIII, extraction of gossypol from defatted seed was erratic regardless of the nature of the solvent or of the moisture content of the seed. Moreover, despite published reports that no gossypol can be detected in Skellysolve F extracts

(*3*, *7*, *14*), it was found, as is shown in Table VIII, that the indicated gossypol content of Skellysolve F–defatted seed is almost invariably less than that of the nondefatted seed. In view of these facts, it is apparent that accurate gossypol determinations can be obtained only by the direct extraction of nondefatted seed with ether or chloroform.

PROCEDURE FOR DETERMINATION OF EXTRACTABLE GOSSYPOL OF COTTONSEED AND COTTONSEED MEAL

REAGENTS. The chloroform and acetic anhydride should be C.P. grade and the antimony trichloride should be anhydrous, C.P. grade.

The saturated chloroform solution of antimony trichloride is prepared as follows: Wash about 30 grams of finely ground antimony trichloride with a small volume of chloroform. Add 100 ml. of chloroform to the washed crystals, warm the suspension, shake it vigorously, and allow it to cool to room temperature.

The gossypol used for the standardization should be purified as previously described (*1*, *2*).

PROCEDURE. A measured volume of chloroform is added to a weighed sample of ground cottonseed kernels or meal. (For the extraction of raw cottonseed of the usual range of gossypol content, 25 ml. of chloroform to 0.25 gram of ground kernels produce an extract which can be used directly in the antimony trichloride test without dilution. For most hydraulic-pressed meals a larger proportion of meal to chloroform must be used.) The flask is stoppered and the mixture is allowed to stand, with occasional shaking, for 24 hours. A sample of the extract is withdrawn from the equilibration mixture in a manner which prevents evaporation and filters the extract—e.g., by covering the tip of a pipet with cotton or by attaching an inverted sintered-glass suction funnel to the end of a pipet. The sample is then shaken vigorously with concentrated hydrochloric acid, about 10 drops per 5 ml. of extract, and the mixture is allowed to stand for 24 hours.

One milliliter of the extract is transferred to a glass-stoppered absorption cell by means of a pipet. One drop of acetic anhydride and 5 ml. of a saturated chloroform solution of antimony trichloride are introduced into the absorption cell and the mixture is thoroughly agitated.

Within 10 to 40 minutes after the reagents are mixed, the transmission of the test solution at 520 mμ is read against that of a blank consisting of 1 ml. of chloroform, 1 drop of acetic anhydride, and 5 ml. of a saturated chloroform solution of antimony trichloride.

The concentration of gossypol in the original seed or meal is calculated by means of the following equation:

$$\% \text{ gossypol} = \frac{\log I_0/I \times V/W}{E1\% \text{ gossypol}}$$

Log I_0/I is the extinction at 520 mμ of the gossypol–antimony trichloride test solution. I_0 is the transmission of the antimony trichloride reagent blank at 520 mμ. I is the transmission of the gossypol–antimony trichloride test solution at 520 mμ. V is the volume of solvent used in the extraction of the seed. W is the weight of cottonseed extracted. $E1\%$ gossypol is the extinction coefficient at 510 to 520 mμ, as previously defined, calculated for 1% gossypol in the original solution before reaction with antimony trichloride. With the absorption cells used in the Coleman double monochromator spectrophotometer $E^{1\%}_{1.27\text{ cm.}} = 65.5 \pm 1.9$.

Table VI. Effect of Moisture on Extraction of Gossypol from Cottonseed

Treatment of Ground Meats[a]	Solvent	*E* at 520 mμ per Gram	Per Cent Gossypol
None	$CHCl_3$	66.3	1.013
H_2O added	$CHCl_3$	61.0	0.932
None	$(C_2H_5)_2O$	66.3	1.013
H_2O added	$(C_2H_5)_2O$	70.4[b]	1.073
None	$CHCl_3$	64.8	0 990
Dried[c]	$CHCl_3$	11.3	0.107
Dried[c]	Wet $(C_2H_5)_2O$[d]	69.5	1.061
Dried[c]	Wet $(C_2H_5)_2O$[d]	65.3	0.998
Dried[c]	Wet $(C_2H_5)_2O$[d]	67.0	1.022
Dried[e]	$(C_2H_5)_2O$	62.9	0.961
Dried[e]	$CHCl_3$	62.9	0.961
Wet[f]	$(C_2H_5)_2O$	65.2	0.996

[a] Original meats contained 8.51% moisture.
[b] Sediment observed in filtered extract.
[c] Dried in desiccator to 3.12% moisture.
[d] Ether contained 1% H_2O and 2.5% C_2H_5OH.
[e] Dried as in [c], then exposed to moist air for 24 hours. Final meats contained 7.57% moisture.
[f] Dried as in [c], then moistened with 2 drops of H_2O per 0.25 gram of ground meats.

Table VII. Effect of Variation in Ratio of Volume of Extraction Solvent to Weight of Cottonseed

Cottonseed Sample	Ml. of $CHCl_3$ per Gram of Meats	*E* at 520 mμ per Gram	Per Cent Gossypol
C-77-VIII	25	62.0	0.947
	50	61.0	0.932
	125	62.0	0.947
C-77-VII	25	63.6	0.971
	50	63.0	0.962
C-77-IX	25	66.3	1.01
	100	64.8	0.990
205e	50	22.8	0.348
	100	22.9	0.350

Table VIII. Extraction of Gossypol from Defatted and Nondefatted Cottonseed Meats

Cottonseed Sample	Treatment of Ground Meats Prior to Equilibration	Per Cent Moisture	Equilibration Solvent	R_a	R_b	*E* at 520 mμ per Gram[a]	Per Cent Gossypol
C-77-VII	None	8.70	$(C_2H_5)_2O$	2.21	1.13	65.5	1.00
	None	8.70	$CHCl_3$	1.59	0.90	64.8	0.991
	None	8.70	$CHCl_3$	1.90	1.01	64.5	0.985
	None	8.70	$CHCl_3$	1.92	1.10	64.5	0.985
	Defatted	12.29	$CHCl_3$	1.57	1.01	30.6	0.467
	Defatted	12.29	$CHCl_3$	1.70	0.97	58.5	0.893
	Defatted	12.29	$CHCl_3$	1.83	0.98	39.1	0.597
	Defatted	12.29	$CHCl_3$	2.14	1.19	39.2	0.598
	Defatted[b]		$CHCl_3$	1.86	1.19	36.1	0.552
	[c]	12.29	$CHCl_3$	1.81	1.13	43.2	0.660
	Defatted	12.29	$(C_2H_5)_2O$	2.10	1.11	39.2	0.598
	Defatted	12.29	$(C_2H_5)_2O$[d]	2.30	1.12	38.1	0.582
C-77-VIII	None	7.17	$CHCl_3$	2.04	1.10	61.0	0.932
	None	7.17	$CHCl_3$	1.85	0.99	62.0	0.947
	None	7.17	$CHCl_3$	1.94	1.03	61.0	0.932
	Defatted	.....	$CHCl_3$	1.97	1.03	61.9	0.945
	Defatted	.....	$CHCl_3$	1.89	1.09	46.8	0.716
	Defatted	.....	$CHCl_3$	1.81	1.07	46.2	0.710
C-77-IX	None	8.51	$CHCl_3$	2.00	1.13	66.3	1.01
	None	8.51	$(C_2H_5)_2O$	2.14	1.18	66.3	1.01
	None	8.51	$CHCl_3$	2.07	1.10	64.8	0.991
	Defatted	12.50	$CHCl_3$	1.79	1.07	42.5	0.648
C-105b	None	.....	$CHCl_3$	2.04	1.17	67.2	1.027
	Defatted	.....	$CHCl_3$	1.82	1.09	41.7	0.637
C-78	None	.....	$CHCl_3$	2.29	1.19	45.2	0.690
	Defatted	.....	$CHCl_3$	1.80	1.13	23.4	0.357

[a] Calculated on basis of meats before defatting.
[b] Five drops H_2O added to 0.5 gram of defatted meats.
[c] Five drops refined cottonseed oil added to 0.5 gram of defatted meats
[d] Ether containing 1% H_2O, 2.5% C_2H_5OH.

SUMMARY

The reaction of gossypol with antimony trichloride in chloroform produces a soluble red product having a characteristic absorption curve in the visible and near ultraviolet region of the spectrum. The absorption curve exhibits a broad, stable maximum at 510 to 520 mμ. The extinction at this absorption maximum is proportional to the concentration of gossypol.

The antimony trichloride reaction is specific for gossypol in ether and chloroform extracts of cottonseed and the reaction product is sufficiently stable to permit the accurate determination of the gossypol concentration of such extracts by means of the spectrophotometer.

The gossypol–antimony trichloride reaction has been used as a means for determining the optimum conditions for the extraction of free gossypol from cottonseed. Chloroform and ether extract equal amounts of gossypol. Equilibration of meats and solvent for 24 hours is adequate for complete solution of extractable gossypol. Moisture is an important factor in the extraction of gossypol only in the case of very dry seeds. The proportions of solvent to meats may be varied within wide limits.

A rapid, direct method for the determination of "free" gossypol in cottonseed has been reported in which the gossypol is extracted by equilibrating ground cottonseed and chloroform. The extract is treated with concentrated hydrochloric acid and the antimony trichloride reaction is applied directly to the treated extract.

The method has been shown to be applicable to the determination of the free gossypol content of hydraulic-pressed meal in which gossypol occurs to a large extent in the form of an orange-

colored pigment which is not precipitated by aniline but is readily converted to gossypol.

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